

the abyssal stations. The degree of horizontal separation seems unrelated to phenotypic divergence. A lesser degree of differentiation occurs among abyssal samples, and it does not appear to correspond in any consistent way to either depth or horizontal separation. Overall, the geographic variation in shell geometry observed is too idiosyncratic and subtle to warrant speculation about its potential adaptive significance. Also, it is important to recognize that we cannot determine the degree to which it represents selection or phenotypic plasticity (Trussell, 1996). The most significant finding is that *X. naticiformis* shows only very modest geographic variation, particularly among abyssal populations, on quite large geographic scales.

Both the relative degree of geographic differentiation in *Xyloskenia naticiformis* and its association with depth rather than horizontal separation accord well with the overall trend in geographic variation observed in other deep-sea prosobranchs of the western North Atlantic (Rex et al., 1988; Rex & Etter, 1990; Etter & Rex, 1990). Intraspecific differentiation in shell form measured as Mahalanobis' generalized distance (D^2) is highest on the upper continental slope and decreases with increasing depth to the abyssal plain (Etter & Rex, 1990). Size-depth clines in gastropod shells also become less pronounced with increasing depth (Rex & Etter, 1998).

Geographic variation in deep-sea species appears to be less well developed than coastal snails show, even on much smaller geographic scales—though most studies of form in shallow-water species focus on a single taxon, the littorinids (cf., e.g., Newkirk & Doyle, 1975; Johannesson 1986; Grahame et al., 1990). Within the deep sea, differentiation tends to be more associated with depth than with horizontal separation (Rex et al., 1988; Rex & Etter, 1990; France & Kocher, 1996). The rate of faunal replacement with depth in snails decreases with increasing depth (Rex, 1977, 1981), and is highly correlated with phenotypic divergence among samples (Etter & Rex, 1990). Both phenotypic change within species and the rate of species turnover reflect the steepness of the environmental gradient which appears to parallel the depth gradient, at least for prosobranchs in the deep western North Atlantic. In contrast to the bathyal environment, the abyssal plain seems to be characterized by a monotonous assemblage of snails and little intraspecific geographic variation in shell architecture on regional spatial scales. Findings presented here for *Xyloskenia naticiformis* support the theory that the abyss is less conducive than the bathyal zone to population differentiation in gastropods of the western North Atlantic (Etter & Rex, 1990; Rex & Etter, 1998).

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Intermating Interval and Number of Sperm Delivered in the Simultaneously Hermaphroditic Land Snail *Arianta arbustorum* (Pulmonata: Helicidae)

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Abstract. The number of sperm delivered is an important determinant for achieving fertilization in sperm competition. Hermaphroditic gastropods with short mating intervals may deplete their autosperm reserves. An earlier study showed that individuals of the simultaneously hermaphroditic land snail *Arianta arbustorum* need at least 8 days to replenish their autosperm reserves after a successful copulation, and that the number of sperm transferred in the second copulation slightly increased up to an intermating interval of 4 weeks. We compared spermatophore size, number of sperm delivered, and mating behavior in snails with longer intermating intervals. Snails that remated after 7–8 weeks did not differ in spermatophore size and number of sperm transferred from individuals that remated after 3–4 weeks. The number of sperm delivered averaged 2,151,000 in the first copulation and 2,130,000 in the second copulation. Snails with a longer intermating interval showed a shorter courtship, but did not differ in copulation duration from snails which remated after 3–4 weeks. Furthermore, different intermating intervals did not affect female fecundity (number of eggs produced and hatching success of eggs). These results indicate that *A. arbustorum* entirely replenishes its autosperm reserves within 3–4 weeks after a successful copulation.

INTRODUCTION

Pulmonate land snails are simultaneous hermaphrodites with internal fertilization. Individuals of a variety of species mate with two or more different partners in the course of a reproductive season and store foreign sperm for long periods. Promiscuous mating and sperm storage are a prerequisite for sperm competition, i.e., the competition between spermatozoa from two or more males to fertilize the eggs of a single female (Parker, 1970). Sperm competition might significantly affect the reproductive biology of pulmonate land snails. However, with a few exceptions, evolutionary and behavioral aspects of sperm competition have not been examined in terrestrial gastropods (Baur, 1998).

Multiple mating is common in helcid snails. Individuals of *Helix pomatia* (Linnaeus, 1758), *Cepaea nemoralis* (Linnaeus, 1758), and *Arianta arbustorum* (Linnaeus, 1758) have been observed to mate repeatedly with different partners in the course of a reproductive season, resulting in multiple-sired broods (Wolda, 1963; Murray, 1964; Baur, 1988). *Helix pomatia* copulated two to six times per year in a Danish population (Lind, 1988), two to four times in a German population (Tischler, 1973), and *H. aspersa* on average three times (maximum seven times) in a British population (Fearnley, 1993, 1996). Paternity analysis in egg batches of *A. arbustorum* indicated

that at least 63% of the snails used sperm from two or more mates for the fertilization of their eggs (Baur, 1994).

The few data available on mating frequency in gastropods suggest that terrestrial gastropods copulate less frequently than freshwater and marine gastropods (Baur, 1998). In intertidal and terrestrial gastropods, the reproductive activity is limited by favorable environmental conditions (the high risk of desiccation may incur a significant cost of mating). Other explanations for the relatively small number of copulations in terrestrial pulmonates include the cost of mucus production during mating, spermatophore production (in some species), as well as the large number of sperm delivered during a copulation which may result in sperm depletion. A previous study showed that individuals of *A. arbustorum* needed at least 8 days to replenish their sperm reserves after a successful copulation (Locher & Baur, 1999). Furthermore, the number of sperm delivered in the second copulation increased with an increasing intermating interval from 6 to 29 days. This finding suggests that the number of sperm delivered increases with even longer intermating interval. The present study examines this idea.

Arianta arbustorum is a simultaneously hermaphroditic land snail common in moist habitats of northwestern and central Europe. The snail has determinate growth (shell breadth of adults 17–22 mm); individuals become sexually mature at an age of 2–4 years, and adults live another 3–4 years (maximum 14 years; Baur & Raboud, 1988). In the field, snails deposit one to three egg batches consisting of 20–50 eggs, per reproductive season (Baur &

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Raboud, 1988; Baur, 1990). Breeding experiments showed that 12 of 44 virgin individuals (27%) prevented from mating produced a few hatchlings by self-fertilization in the second and third years of isolation (Chen, 1993). The reproductive success of selfing individuals, however, was less than 2% of that of mated snails, suggesting high costs for selfing (Chen, 1994).

Mating behavior in *A. arbustorum* includes elaborate courtship behavior with optional dart shooting, i.e., the pushing of a calcareous dart into the mating partner's body, and lasts 2–18 hr (Hofmann, 1923; Baur, 1992a). Copulation is reciprocal; after intromission each snail transfers simultaneously one spermatophore (Haase & Baur, 1995). The spermatophore is formed and filled with sperm during copulation (Hofmann, 1923). It has a distinctive form consisting of a head, a body (sperm container with 800,000–4,000,000 spermatozoa) and a tail 2–3 cm long (Baur et al., 1998; Locher & Baur, 2000). The snails mate repeatedly in the course of a reproductive season, and fertile sperm can be stored for more than 1 year (Baur, 1988). Mating was found to be random with respect to shell size and different degrees of relatedness (Baur, 1992a; Baur & Baur, 1997). A controlled laboratory experiment showed that one successful copulation per reproductive season is sufficient to fertilize all the eggs produced by an individual (Chen & Baur, 1993). However, there is a probability of 5–8% that a copulation will not lead to fertilization of eggs (no sperm transfer or transfer of unfertile sperm; Chen & Baur, 1993).

In the present study we examined whether *A. arbustorum* that remated after 7–8 weeks delivered more sperm than snails remating after 3–4 weeks. We also investigated whether sperm delivery is influenced by courtship and mating behavior in this snail species.

MATERIALS AND METHODS

Maintenance of Test Snails

To obtain virgin *A. arbustorum*, subadult individuals that had not yet completed shell growth were collected in a subalpine forest near Gurnigelbad, 30 km south of Bern, Switzerland (46°45'N, 7°27'E; at an elevation of 1320 m above sea level) on 13 May 1999. The snails were kept isolated in transparent plastic beakers (8 cm deep, 6.5 cm in diameter) lined with moist soil mixed with powdered limestone (approximately 4 cm) at $19^{\circ} \pm 1^{\circ}\text{C}$ and with a light/dark cycle of 16:8 hr for 7 weeks. During this period, subadult individuals reached sexual maturity as indicated by the formation of a flanged lip at the shell aperture. Fresh lettuce was provided *ad libitum* as food. The beakers were cleaned twice per week.

Mating Trials

Mating trials were performed outdoors to expose snails to natural temperature and light conditions. Two random-

ly chosen active snails (individuals with an extended soft body and everted tentacles) were allowed to copulate in a transparent plastic container, measuring $14 \times 10 \times 7$ cm, whose bottom was covered with moistened paper towels to maintain activity. One of the two snails was marked on its shell with a spot of correction fluid (Tipp-Ex®) to be able to distinguish between the two partners when recording their behavior. The animals showed no visible reaction to the marking procedure. Mating trials were initiated in the late evening (after 10 p.m.) and ran during 14 nights in June (first copulations) and July and August 1999 (second copulations). The period between the end of May and the middle of July is the time of maximum mating activity in subalpine populations of *A. arbustorum*.

The snails' mating behavior was observed at intervals of 30 min (at night using a flashlight) following the method described in Baur (1992a) and Baur et al. (1998). Records included time until initiation of courtship, courtship duration (time interval from courtship initiation to copulation), and copulation duration. The initiation of courtship was defined as the first simultaneous oral contact, which was usually accompanied by a slight eversion of the penial lobe in one of the snails. The beginning of copulation was defined as the first simultaneous penis intromission. Observation sessions were terminated either when two snails copulated or after 6 hr if no snail initiated courtship behavior in a test arena. Snails that did not mate were tested again 3–7 days later with a new partner. In the period between two trials, the snails were kept isolated as described above. In all, 39 copulations were observed in 177 trials (22.0% successful trials).

After copulation, one mating partner (hereafter referred to as sperm donor) was kept isolated in a transparent plastic beaker lined with moist soil (as described above). The other mating partner (hereafter referred to as sperm recipient) was frozen immediately after copulation.

To assess the influence of the interval between two copulations on the number of sperm delivered, sperm donors were allowed to remate with a randomly assigned virgin partner either 3–4 weeks or 7–8 weeks after the first copulation (Figure 1). One sperm donor of the first group died before the second mating. Seven sperm donors remated after 3–4 weeks (7 copulations in 99 trials; 7.1% successful trials) and 11 sperm donors after 7–8 weeks (11 copulations in 107 trials; 10.3%). In the latter group, two snails did not deliver any spermatophore in the second copulation. These animals were omitted in the data analyses, reducing the sample size of this group to nine.

To assess any size effect of the sperm donor on the number of sperm transferred and number of eggs produced, we measured the size (shell breadth and height) of each mating snail to the nearest 0.1 mm using vernier calipers and calculated the shell volume using the formula:

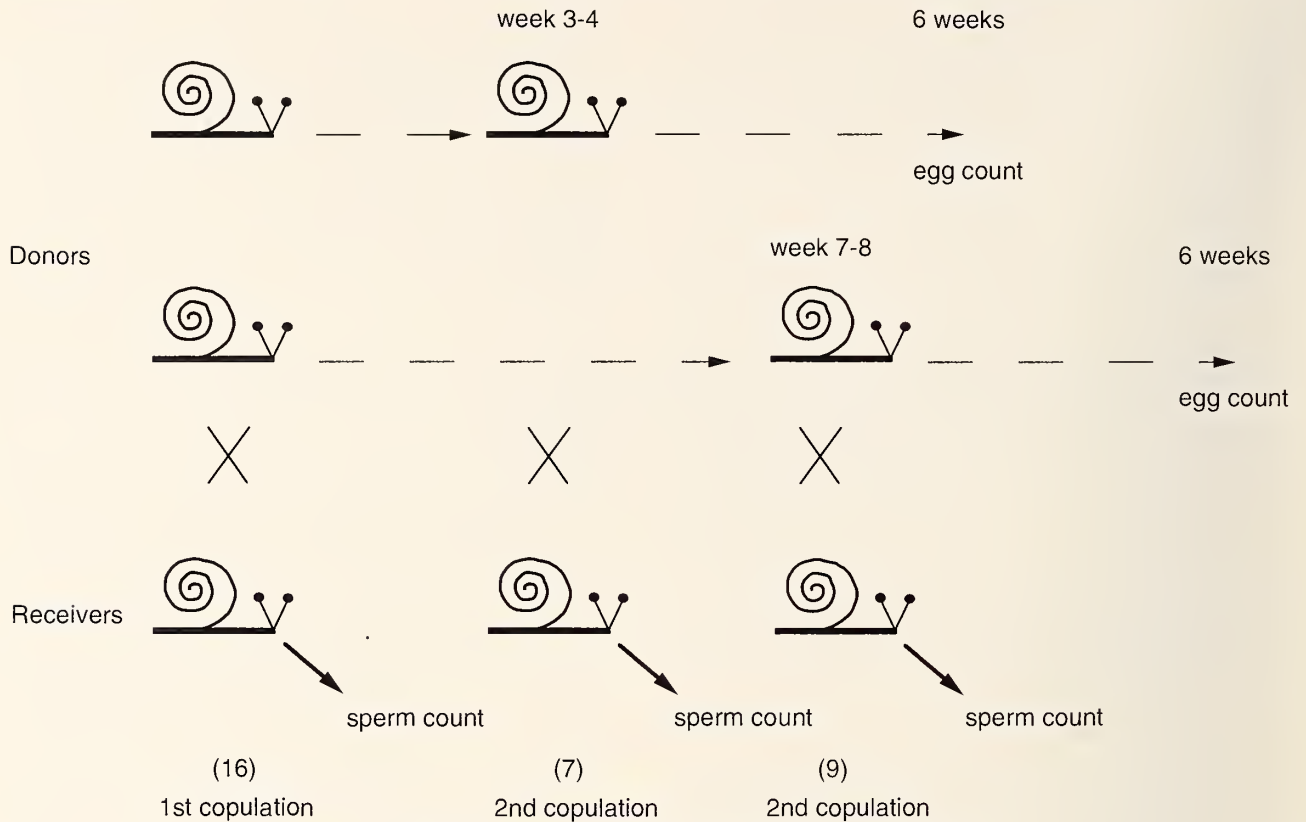


Figure 1. Design of mating experiment with sample size in parentheses.

shell volume = $0.312 \times [(\text{breadth})^2 \times \text{height}] - 0.038$ (measurements in mm; B. Baur, unpublished data). Shell volume is a more reliable measurement of snail size than weight because weight depends on the state of hydration and thus is highly variable in terrestrial gastropods.

To obtain the spermatophore we dissected out the female reproductive duct of the recipient. The length (L) and width (W) of the sperm-containing part of each spermatophore were measured to the nearest 0.1 mm using a dissecting microscope. Spermatophore size (in mm^3) was approximated, by the formula ($\pi LW^2/4$), assuming a cylindrical volume. Spermatophores were kept singly in Eppendorf tubes at -30°C until required.

The beakers of sperm donors were checked twice per week for eggs. The eggs of each batch were collected, counted, and kept in a plastic dish (6.5 mm in diameter) lined with moist paper towels at $19^\circ \pm 1^\circ\text{C}$ to determine hatching success. Newly hatched snails were separated from remaining unhatched eggs to prevent egg cannibalism (Baur, 1992b). In both groups of snails, eggs were collected over a period of 40 days following the second copulation.

Sperm Counting Procedure

The number of sperm that an individual delivered was assessed by counting the number of sperm in the sper-

matophore transferred. This procedure is described in detail by Locher & Baur (1997). The spermatophore of *A. arbustorum* consists of a hardened secretion which encapsulates the spermatozoa (Hofmann, 1923). We mechanically disrupted the spermatophore in 200 μl PBS-buffer (138.6 mM NaCl, 2.7 mM KCl, 8.1 mM $\text{Na}_2\text{HPO}_4 \times 2\text{H}_2\text{O}$ and 1.5 mM KH_2PO_4) using a pair of microscissors. The sperm suspension was homogenized with a set of Gilson pipettes for 5–15 min. To count the sperm, the homogenate was stained for 1–3 hr with an equal volume of a gallocyanin-chromium complex which stains the DNA in the head of the spermatozoa. If spermatozoa still occurred in clusters, we treated the sample overnight with a sonicator (35 kHz). Two subsamples of known volume of the sperm suspension were diluted 1:3 with PBS-buffer and transferred to a Bürker-Türk counting chamber. This counting chamber consists of 16 cells each with a volume of 25 nL. We counted all sperm heads in randomly chosen cells until the total number of sperm heads exceeded 400 and used the average of two subsamples to calculate the total number of sperm in a spermatophore.

Data Analyses

The StatView program package (Version 5.0, Abacus Concepts, 1998) was used for statistical analyses. Means

Table 1

Mating behavior, sperm delivery, and female fecundity in *A. arbustorum* that remated either after 3–4 weeks or after 7–8 weeks. Data from the second copulation are shown (egg number, hatching success of eggs, and number of hatchlings relate to the entire experimental period). Mean values \pm SE are presented. *P*-values result from unpaired *t*-tests.

Trait	Length of intermating interval		<i>t</i>	<i>P</i>
	3–4 weeks (<i>n</i> = 7)	7–8 weeks (<i>n</i> = 9)		
Time until initiation of courtship (min) ¹	107 \pm 14	133 \pm 33	0.17	0.86
Courtship duration (min) ¹	416 \pm 45	263 \pm 28	3.09	0.008
Copulation duration (min) ¹	120 \pm 9	130 \pm 13	0.49	0.63
Spermatophore volume (in % of spermatophore size in the first copulation)	87.0 \pm 10.3	99.4 \pm 6.3	1.07	0.30
Sperm (in % of sperm in the first copulation)	106.1 \pm 18.1	110.2 \pm 17.4	0.16	0.88
Total number of eggs produced ¹	64.4 \pm 20.0	50.0 \pm 10.1	1.23	0.24
Hatching success (%) ²	64.7 \pm 11.1	72.1 \pm 7.4	0.70	0.50
Total number of hatchlings ¹	48.4 \pm 15.4	38.3 \pm 9.2	1.22	0.24

¹ \log_{10} -transformed.

² *arcsine*-transformed.

\pm 1 SE are given unless otherwise stated. We only considered snails that copulated twice and set the significance level α at 0.01 to compensate for the large number of statistical tests based on data of the same individuals. To improve normality, some variables were \log_{10} - or *arcsine*-transformed.

RESULTS

First Copulation

The size of the spermatophore delivered during the first copulation varied from 1.93 to 3.84 mm³ ($\bar{x} \pm$ SE = 2.74 \pm 0.16 mm³, *n* = 16). The number of sperm transferred in the first copulation ranged from 1,281,800 to 3,599,700 (2,151,000 \pm 165,600, *n* = 16) and tended to be positively correlated with the size of the spermatophore (r = 0.53, *n* = 16, P = 0.032). Furthermore, spermatophore size tended to be positively correlated with the shell size of the sperm donor in the first copulation (r = 0.51, *n* = 16, P = 0.044). However, no correlation was found between number of sperm delivered and the shell size of the sperm donor (r = 0.04, *n* = 16, P = 0.89). Similarly, no correlation was found between spermatophore size, respectively, sperm number and the shell size of the sperm recipient in the first copulation (spermatophore size: r = 0.49, *n* = 16, P = 0.0513; sperm number: r = 0.16, *n* = 16, P = 0.56).

Virgin snails needed 105 min (median, range 30–360 min, *n* = 16) to initiate courtship. The median courtship time was 285 min (range 180–540 min, *n* = 16) and the median copulation duration was 150 min (range 90–300 min, *n* = 16). Neither courtship nor copulation duration was significantly correlated with the number of sperm transferred in a spermatophore (Spearman rank correla-

tion: courtship r_s = 0.27, *n* = 16, P = 0.29; copulation r_s = -0.14, *n* = 16, P = 0.59).

Effect of Intermating Interval

Snails that remated after 3–4 weeks did not differ in shell size from those that remated after 7–8 weeks (mean shell volume of both groups: 1.24 cm³, range: 1.07–1.39 cm³; t = 0.09, *df* = 14, P = 0.93). There were differences in mating behavior between the two groups of snails. Courtship duration was shorter in individuals that remated after 7–8 weeks than in snails that remated after 3–4 weeks (Table 1). However, time until initiation of courtship and copulation duration did not differ between snails of both groups (Table 1). Furthermore, mating propensity (percentage of snails that mated in the trials) did not differ between the two groups (7.1% vs. 10.3%; χ^2 = 0.66, *df* = 1, P > 0.4). Compared with the first copulation, however, the average mating propensity was lower in the second copulation (8.7% vs. 22.0%; χ^2 = 13.29, *df* = 1, P < 0.001).

The difference in intermating interval did not affect spermatophore size and the number of sperm delivered in the second copulation (Table 1). Furthermore, individuals of neither treatment group differed in the number of sperm delivered in the first and second copulation (paired *t*-test; intermating interval 3–4 weeks, t = 0.17, *df* = 6, P = 0.87; intermating interval 7–8 weeks, t = 0.25, *df* = 8, P = 0.81). The number of sperm transferred in the second copulation averaged 2,130,000 (range 1,014,900–3,537,600, *n* = 16). No correlation was found between number of sperm delivered in the second copulation and the shell size of the donor and that of the receiver (donor: r = 0.20, *n* = 16, P = 0.46; receiver: r = 0.12, *n* = 16, P = 0.66). Furthermore, snails of both treatment groups

did not differ in female reproductive success (number of eggs produced and hatching success of eggs; Table 1). Moreover, female reproductive success did not differ between the two groups of snails when only a period of 40 days following the second copulation is considered (*t*-tests, in all traits $P > 0.36$, data not shown).

DISCUSSION

The present study showed that the number of sperm transferred in a copulation of *A. arbustorum* did not increase when the intermating interval was prolonged from 3–4 to 7–8 weeks. This finding supplements the results of a previous study which indicated that individuals of *A. arbustorum* require at least 8 days to replenish their sperm reserves after a successful copulation, and that there is a slight increase in number of sperm delivered in the second copulation when the intermating interval extends to 22–29 days (Locher & Baur, 1999).

In simultaneously hermaphroditic opisthobranchs and pulmonates, the ovotestis produces both spermatozoa and ova, sometimes but not always simultaneously (Duncan, 1975). In *Helix pomatia*, autosperm are stored in the seminal vesicle of the hermaphrodite duct throughout the year (Lind, 1973). Phagocytosis of autosperm by the hermaphrodite duct epithelium has been reported in *H. pomatia* and *Oxychilus cellarius* (Müller, 1774) (Rigby, 1963). Sperm can be expelled from the hermaphrodite duct at times other than copulation to be eventually digested (as are foreign sperm) in the bursa copulatrix. In this way, unfertile and old spermatozoa can be recycled.

In many animal species, sperm number is an important determinant for achieving successful fertilization in sperm competition (Birkhead & Möller, 1998). In gastropods with internal fertilization, sperm are transferred to the partner in the form of free sperm, i.e., as sperm suspension in seminal plasma, or the sperm are either aggregated into loosely assembled naked conglomerates (spermatozeugmata) or encapsulated into spermatophores (Mann, 1984). However, little information is available about the number of sperm delivered in different gastropod species. In the sea hare *Aplysia parvula* Guilding in Mörch, 1863, the number of sperm transferred is positively correlated with copulation duration. When mating duration increased from 2 to 47 min, the number of sperm transferred increased from 1×10^5 to 6×10^6 (Yusa, 1994). In *Aplysia kurodai* Baba, 1937, and *A. juliana* Quoy & Gaimard, 1832, the number of fertilized eggs laid by an individual, which was allowed to mate only once, is positively correlated with copulation duration (Yusa, 1996). The ratio of transferred sperm to fertilized eggs is approximately 30:1 (Yusa, 1996).

Most freshwater pulmonates transfer a seminal fluid in which sperm is embedded (Geraerts & Joosse, 1984). During one copulation, the freshwater pulmonate *Bulinus globosus* ejaculates at least 350,000 sperm (Rudolph,

1983). *Bulinus globosus* (Morelet, 1866) is able to copulate as male once per day for up to 8 consecutive days. Following a single copulation, after 1 week of isolation, the hermaphroditic duct of male-acting individuals contained an average of $87,000 \pm 42,000$ (SD) sperm. In the 10 days following the initial copulation, the snails produced approximately 50,000 sperm per day.

Arianta arbustorum transfers its spermatozoa in spermatophores. The ratio of transferred sperm to fertilized eggs is approximately 50,000:1, if one copulation is considered, or 25,000:1, if two copulations are considered. These figures significantly exceed the corresponding ratio recorded in *Aplysia* (see above). The present estimates of sperm number coincide with estimates of two independent studies using snails from the same subalpine population: the number of sperm delivered averaged 2,185,100 ($n = 91$, range 802,620–3,968,800) in Baur et al. (1998) and 2,573,000 ($n = 31$, range: 907,000–5,825,000) in Locher & Baur (1999). In contrast, lower numbers of sperm were transferred in three *A. arbustorum* populations in the Austrian Alps (mean values: 1,707,000 ($n = 14$), 1,615,000 (15), and 1,802,000 (14), respectively; Baminger et al., 2000). However, the latter estimates were obtained from snails copulating in the wild, while higher sperm numbers were observed in animals kept under laboratory conditions. It is possible that geographical variation in number of sperm delivered exists in *A. arbustorum*. The production of sperm will certainly vary depending on the environment, the age, size, and nutritional state of the snail and most probably on the level of sperm competition.

In the present study, snails showed a higher mating propensity when they were allowed to copulate for the first time in June (22.0%) than when they were allowed to remate in July or August (8.7%). We used the percentage of individuals that mated in the trials as a measure of mating propensity. This is an indirect measure of mating frequency. A previous study showed that the more active a snail is, the more likely it will initiate courtship (Baur & Baur, 1992). Using similar experimental procedures, mating propensity ranged from 10.0% to 33.3% in different *A. arbustorum* populations (Baur & Baur, 1992). In natural populations, mating frequency decreases after the peak period (May to June). A similar seasonal decrease in mating propensity was observed in the present experiment. Most interestingly, snails prevented from remating for 7–8 weeks showed a shorter courtship duration than those prevented for 3–4 weeks. This difference cannot be explained by different conditions in the mating trials between the two experimental groups, as air temperature was similar during the test nights (Hänggi, 2000). On the other hand, seasonal effects on courtship duration cannot be ruled out. Courtship duration might also be short if there is no (or little) conflict between the gender roles in hermaphroditic mating partners (see Michiels, 1998). In *A. arbustorum*, copulations toward the